

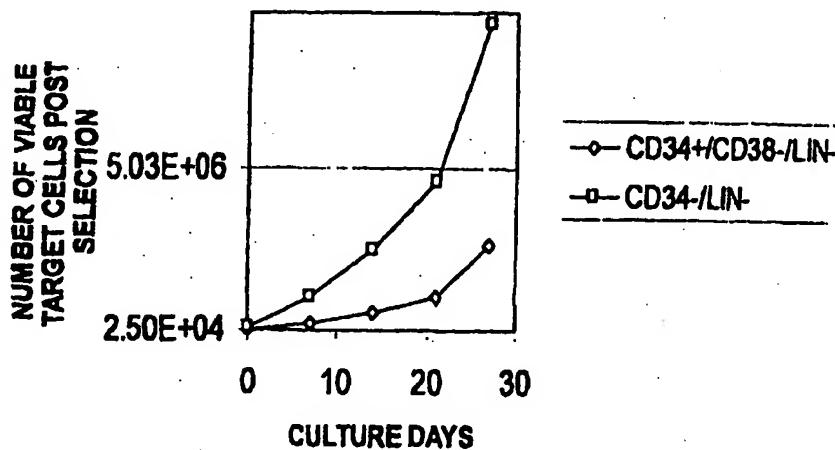


INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 5/08, 5/00		A1	(11) International Publication Number: WO 00/11139 (43) International Publication Date: 2 March 2000 (02.03.00)
(21) International Application Number: PCT/US99/19159 (22) International Filing Date: 24 August 1999 (24.08.99) (30) Priority Data: 09/138,928 24 August 1998 (24.08.98) US		(81) Designated States: CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(71) Applicant: T.BREEDERS, INC. [US/US]; One Innovation Drive, Worcester, MA 01605 (US). (72) Inventors: KRAUS, Morey; 167 Richmond Avenue, Worcester, MA 01602 (US). WILDER, Paul; 11 Chicopee Street, Worcester, MA 01602 (US). (74) Agent: LEBER, Celia, H.; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110-2804 (US).			

(54) Title: CELL POPULATION CONTAINING NON-FETAL HEMANGIOBLASTS AND METHOD FOR PRODUCING SAME

**GROWTH KINETICS FOR CD34+/CD38-/LIN- and
CD34-/CD38-/LIN- TARGET CELLS**



(57) Abstract

Methods are provided for expanding populations of non-fetal hemangioblasts, for example a method including (a) providing a first cell population containing non-fetal hemangioblasts; and (b) growing the enriched cell culture under conditions that promote the proliferation of the non-fetal hemangioblasts. Preferred populations includes non-fetal uncommitted human hemangioblasts.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Larvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

- 1 -

CELL POPULATION CONTAINING NON-FETAL HEMANGIOBLASTS
AND METHOD FOR PRODUCING SAME

5 Background of the Invention

The invention relates to cell populations and methods of producing them.

There is strong empirical evidence that during murine embryogenesis a common precursor to hematopoietic and endothelial cells exists. A pluripotent precursor cell, called the hemangioblast, which carries this bipotential, was first hypothesized in 1900 by W. His. Putative hemangioblasts have been teased out of embryonic cultures and manipulated by cytokines to differentiate 10 along either hematopoietic or endothelial pathways. Using population kinetics it has been demonstrated that pre-endothelial/pre-hematopoietic cells in the embryo clearly arise out of a phenotype CD 34- population. (Choi et al., A Common Precursor for Hematopoietic and Endothelial Cells, *Development* 125, 725-732 (1998).)

Additionally, it has recently been found that sheep can be successfully engrafted *in utero* with adult human CD34⁺, Lin⁻ cells, resulting in long-term engraftment and multi-lineage hematopoietic 15 cell/progenitor expression. Significant numbers of human CD34⁺ cells were detected in the animals that were transplanted with the CD34⁺, Lin⁻ cells. The resulting conclusion was that the CD34⁺ fraction of normal human bone marrow contains cells capable of enabling *in utero* 20 engraftment, possibly through the differentiative production of engraftable CD34⁺ cells in the fetal microenvironment. (Zanjani et al., Human Bone Marrow CD34- Cells Engraft *In Vivo* and Undergo Multilineage Expression That Includes Giving Rise to CD34⁺ Cells, 25 Experimental Hematology 26:1-221 (1998).)

- 2 -

Summary of the Invention

The invention provides enriched stem cell containing populations that can be expanded, and thus can be of great value to patients in need of cellular 5 therapy, e.g., cancer therapy, immunotherapy, and gene therapy.

Preferred populations include at least 5% human non-fetal uncommitted hemangioblasts, i.e., common precursors of hematopoietic cells and endothelial cells; 10 by "uncommitted" it is meant that the hemangioblasts are not yet committed to either lineage, i.e., under the proper conditions the cells can become either hematopoietic cells or endothelial cells. These hemangioblasts are stable, not transient, and are present 15 in the tissue of fully developed individuals, such as in newborn infants and adults. We have, in fact, discovered that these hemangioblasts can be isolated from cord blood following birth. The presence of hemangioblasts in non-embroid tissue was unexpected and presents novel 20 opportunities.

As shown schematically in Fig. 1, the uncommitted hemangioblasts can be stimulated to become hematopoietic cells or endothelial cells, by selecting appropriate growth factors in which to expand the population, as will 25 be discussed in detail below. Thus, when supplied with one cocktail of growth factors, these hemangioblasts can be amplified (i.e., the number of hemangioblasts can be increased), and/or they can be differentiated to provide a supply of hematopoietic cells, for example to patients 30 who are immune compromised or require gene therapy with hematopoietic cells. When supplied with a different cocktail, the hemangioblasts can be amplified and/or can be differentiated to become endothelial cells, useful for example in wound healing, e.g., healing of slow or non- 35 healing diabetic sores. The endothelial cells can also

- 3 -

be transfected *ex vivo*, e.g., with genes which produce angiogenic factors, and used in gene therapy, for example to stimulate angiogenesis in patients with vascular or cardiac insufficiency. Recent studies have demonstrated
5 the feasibility of cytokine gene transfer to enhance the antitumor activities of host immune cells. Endothelial cells forming the vascular supply of tumors may be useful vehicles for the delivery of cytokine molecules in order to effect tumor immunotherapy. Ojeifo, et al., Cytokines
10 Mol Ther 1996 Jun;2(2):89-101.

Populations which have been expanded to contain a significant percentage of these uncommitted hemangioblasts will provide a high level of engraftment while starting from a relatively small sample, since the
15 uncommitted hemangioblasts can be stimulated to become hematopoietic cells by supplying them with appropriate growth factors.

Accordingly, in one aspect, the invention features a method for providing a cell population containing non-fetal hemangioblasts. The method includes (a) providing a first cell population containing non-fetal hemangioblasts; and (b) growing the first cell population under conditions that promote the proliferation of the non-fetal hemangioblasts. The invention also features
25 cell populations formed by expansion of a population containing non-fetal hemangioblasts.

Preferred embodiments of the invention include one or more of the following features. In the growing step (step (b), above), the conditions are such that the
30 number of said non-fetal hemangioblasts and their proximity to each other are sufficient to increase the proportion of non-fetal hemangioblasts in the population. The method includes, prior to the growing step, enriching the first cell population for non-fetal hemangioblasts.
35 The method also includes separating the non-fetal

- 4 -

hemangioblasts from other cells in the cell culture, e.g., by a negative selection process. The separating step is performed concurrently with, intermittently during, or following, the growing step. The separating

5 step is performed more than once during cell proliferation, e.g., every 5 to 10 days. The growing step includes providing at least one growth factor, more preferably a cocktail of growth factors, to the cell population during cell proliferation. At least some of

10 the non-fetal hemangioblasts, preferably at least 2%, more preferably at least 5%, more preferably at least 15% and most preferably at least 25%, are uncommitted human hemangioblasts. At least some of the non-fetal hemangioblasts, preferably at least 2%, more preferably

15 at least 5%, more preferably at least 15% and most preferably at least 25%, are CD 34-, Lin- cells. The percentage of cells that are CD 34-, Lin- and/or are uncommitted human hemangioblasts is higher in the enriched cell culture than in the starting cell culture.

20 The uncommitted human hemangioblasts are characterized as: CD2⁻, CD3⁻, CD14⁻, CD16⁻, CD19⁻, CD24⁻, CD56⁻, CD66b⁻, glycophorin A⁻. The uncommitted human hemangioblasts are further characterized as: flk-1⁺, CD45⁺, CXCR4⁺, MDR+ (Pgp).

25 In another aspect, the invention features an enriched cell population comprising non-fetal hemangioblasts, the enriched cell population resulting from expansion of a starting cell population containing fewer non-fetal hemangioblasts than the enriched cell

30 population.

Preferred embodiments include one or more of the following features. The starting cell population contains at least 10% fewer non-fetal hemangioblasts than the enriched cell population. The percentage of cells

35 that are non-fetal hemangioblasts in the enriched cell

- 5 -

culture is the same as or higher than the percentage of cells that are non-fetal hemangioblasts in the starting cell culture.

The invention also features a composition of cells 5 in which at least 2%, more preferably at least 5%, more preferably at least 15%, and most preferably at least 25% of the cells are non-fetal hemangioblasts, and methods of making such a composition. Preferably the non-fetal hemangioblasts are human uncommitted hemangioblasts that 10 are Lin⁻ cells and are characterized as: CD2⁻, CD3⁻, CD14⁻, CD16⁻, CD19⁻, CD24⁻, CD56⁻, CD66b⁻, glycophorin A⁻, flk-1⁺, CD45⁺, CXCR4⁺, MDR⁺.

Other features and advantages of the invention will be apparent from the following detailed description, 15 the drawings, and the claims.

Brief Description of the Drawings

Fig. 1 is a schematic diagram illustrating that hemangioblasts are the common precursor of hematopoietic and endothelial cells.

20 Fig. 2 is a highly enlarged diagrammatic view of a system for positive selection of a target cell (a hemangioblast). Fig. 2a is a highly enlarged diagrammatic view of a system for negative selection of a non-target cell (a non-hemangioblast).

25 Figs. 3-3d are schematic diagrams of alternative modes of operation of systems according to different embodiments of the invention.

Fig. 4 is a schematic flowchart showing a method, according to one embodiment of the invention, of treating 30 a patient with hematopoietic stem cells.

Fig. 5 is a graph showing the growth kinetics of CD34+/CD38-/Lin- and CD34-/CD38-/Lin- target cells over a 30 day culture period.

- 6 -

Fig. 6 is a group of dot plots showing the population profile of reselected cells prior to subsequent reselection using CD34 vs. Lineage (Lin) markers over a three week period.

5 Fig. 7 is a dot plot showing the population profile of initial negatively selected target populations used to inoculated cultures.

Fig. 8 is a group of dot plots showing the population profile of reselected cells at week three of 10 culture using CD45 vs. MHC I and MHC II and CD34 vs. Flk-1.

Fig. 9 is a group of dot plots showing the population profile of reselected cells at week three of 15 selective culture using CXCR4 vs. Flk-1 and Pgp (MDR) vs. Flk-1.

Description of the Preferred Embodiments

As discussed above, the invention broadly features a method of enriching the precursor cell content of a population containing non-fetal hemangioblasts (hereafter referred to as "target cells"). Preferably the cells are CD 34-, Lin- cells or uncommitted hemangioblasts that are characterized as: CD2⁻, CD3⁻, CD14⁻, CD16⁻, CD19⁻, CD24⁻, CD56⁻, CD66b⁻, glycophorin A⁻, flk-1⁺, CD45⁺, CXCR4⁺, MDR⁺.

Selection

25 Preferred methods of expanding and enriching the population include selecting target cells from non-target cells in the cell population, concurrently with proliferation, intermittently during proliferation or following proliferation. Cell proliferation and cell 30 selection can be carried out using an almost infinite variety of different techniques and settings, of which only a few are described below by way of example. Many other techniques will be readily perceived by those

- 7 -

skilled in the art, for example selection by flow cytometry, and selection by using chemical agents to kill unwanted cells.

The preferred selection methods used in the
5 invention can broadly be classed as positive selection
(providing a selection element having an affinity for,
i.e., "selecting", target cells) and negative selection
(providing a selection element having an affinity for,
i.e., "selecting", non-target cells). These two
10 selection techniques, used alone or in combination, allow
unwanted cells to be removed from the system and target
cells to be harvested whenever desired.

An example of a positive selection technique is
illustrated diagrammatically in FIG. 2. Briefly, one or
15 more anti-dextran conjugated antibodies specific for the
predetermined target population is introduced into the
culture. After a specified incubation time, a magnetic
dextran iron particle colloid is introduced into the cell
suspension. A Cell/Antigen/Antibody/Anti-
20 dextran/Dextran/Iron Complex forms. This complex is then
passed through a magnetic field. Positively selected
cells remain in the magnetic field while cells which do
not have the iron conjugated complex are removed. After
capture and rinsing the magnetic field is removed and the
25 positively selected predetermined target population is
returned to the nutrient medium.

An example of a negative selection technique is
illustrated diagrammatically in FIG. 2A. Briefly, one or
more anti-dextran conjugated antibodies specific for a
30 predetermined population which is not of the
predetermined target population is introduced into the
culture. After a specified incubation time, a magnetic
dextran iron particle colloid is introduced into the cell
suspension. A Cell/Antigen/Antibody/Anti-
35 dextran/Dextran/Iron Complex forms. This complex is then

- 8 -

passed through a magnetic field, removing cells not of the predetermined target population from the nutrient medium. The predetermined target population is collected downstream and returned to the nutrient medium.

5 Clearly, many other techniques can be utilized for both positive and negative selection, as long as the desired affinity is provided by the selection element.

The selection element can include other components in addition to the antibody molecules that are used to
10 perform the selection (the "selection molecules"), e.g., a solid support to which the selection molecule is bound. The solid support can be formed of a material that will aid in performing the selection or in maintaining the selection molecules in a desired position or introducing
15 and removing them from the system. For example, as described above with reference to FIG. 2, the selection molecule can be bound to iron or other magnetic particles to allow the selected cells to be easily removed from the system by application of a magnetic field and then
20 collected by removal of the magnetic field.

Alternatively, the selection molecules can be bound onto the wall of a vessel containing the nutrient medium, or of a chamber through which the nutrient medium flows during the method. Glass or other inert, impermeable
25 beads can also be used as a solid support. If beads or other particles are used, they can be provided in a packed configuration, through which the nutrient medium flows, or can be introduced into the system in a loose form, suspension, or in any desired type of array. As
30 will be readily understood, a wide variety of other solid supports can be used.

As shown in FIGS. 3-3D, the selection element can be used in a variety of modes of operation in which nutrient media is supplied to and removed from the system
35 in different manners. These modes of operation range

- 9 -

from a selective batch culture (FIG. 3), in which nutrient media is supplied at the beginning of cell proliferation and is neither added to nor removed, to continuous flow or recycled flow cultures (FIGS. 3C and 5 3D, respectively) in which either fresh or recycled nutrient media flows through the system substantially continuously. These alternative modes will be discussed in detail below.

In a selective batch culture (FIG. 3), a nutrient medium is introduced into a vessel, and a starting sample of cells is also introduced into the vessel. During cell proliferation, nutrient medium may or may not be exchanged. However, selected cells are physically selected, i.e., separated from other cells in the 15 nutrient medium by binding to a selection element, either continuously, intermittently or following cell proliferation. These selected cells may be cells of a target population, if positive selection is used, or unwanted cells, if negative selection is used. Dual 20 (positive and negative) selection can be accomplished by providing positive selection molecules on the surface of the vessel, beads, baffles, impellers, etc. while removing unwanted cells by negative selection. Alternatively, cells may be positively or negatively 25 selected outside of the culture vessel and then returned.

The selective semi-batch (3A) and selective fed batch (3B) modes of operation are similar to the selective batch mode with regard to positive and negative selection. The significant difference between these 30 three modes is in the treatment of the nutrient medium. While in the batch mode the volume of the medium remains constant and the medium is not refreshed (it may be supplemented), the semi-batch mode allows for a partial refreshment of spent medium with new medium and the fed

- 10 -

batch mode allows for an incremental increase in the medium volume over time.

Cell growth and selection can also be performed in a continuous (FIG. 3C) or recycling (FIG. 3D) mode of 5 operation. In these two modes, the system includes a chamber having an inlet and an outlet, and nutrient media is caused to flow through the chamber from the inlet to the outlet. In continuous mode, new nutrient media flows through the chamber from a source or reservoir, while in 10 recycling mode the same nutrient media is cycled through the chamber repeatedly. If desired, a system can be configured to be run alternatively in either continuous or recycling mode. Any desired selection element can be used in these modes of operation.

15 Process Parameters

A number of parameters can be varied to affect the rate and purity of the cell output obtained during cell proliferation.

For example, the concentration, type and 20 combination of growth factors can be varied to achieve a desired result. Suitable growth factors for promoting cell proliferation include Stem Cell Factor (SCF; R&D Systems Catalog No. 255-SC-010), Thrombopoietin (TPo; R&D Systems Catalog No. 288-TP-005) and FLT3 (R&D Systems 25 Catalog Nos. 308-FK-005 and 308-FK-025), all of which are commercially available from R&D Systems, Inc., 614 McKinley Place NE, Minneapolis, MN 55413. Preferably at least 10 ng/ml of each of these growth factors is added, more preferably 10-500 ng/ml. If the cell population 30 contains uncommitted hemangioblasts, the growth factors supplied will determine whether the hemangioblasts become hematopoietic cells or endothelial cells. To obtain hematopoietic cells, the hemangioblasts can be supplied with a cocktail containing SCF, TPo and FLT3; to obtain

- 11 -

endothelial cells the hemangioblasts can be supplied with a cocktail containing VEGF. Suitable concentrations of SCF, TPO and FLT3 are given above. Suitable concentrations of VEGF are from 5 to 100 ng/ml.

5 We have also discovered that proliferation of stem cells is influenced by the proximity of the cells to each other in the culture. We found that proliferation of the target cells is enhanced by periodically increasing the relative concentration of the target cells in the culture

10 during proliferation. The concentration can be increased, for example, by separating out non-target cells and then placing the remaining target cells in closer proximity to each other, e.g., by decreasing the cross-sectional diameter of the culture vessel.

15 If separation is performed, as discussed above, the duration of the time periods between separations will affect cell proliferation. The removal of non-target cells from the culture promotes cell proliferation by reducing build-up of by-products and inhibitors, and thus

20 frequent selection will result in enhanced proliferation of the target population. In most cases, it is preferred that the selection remove substantially all of the non-target cells, as this will minimize inhibition. However, in some cases it may be desirable to allow other

25 populations to proliferate with the target population. Moreover, selection can be eliminated completely if a mixed population can be used or would be desirable in the intended application, e.g., bone marrow restoration where a Graft vs. Leukemia (GVL) type effect is desired.

30 Other factors that will influence the composition of the cell population resulting from proliferation include the nutrient media used, the gas tension in the incubator, the seeding density (initial concentration of non-fetal hemangioblasts), and stirring of the culture.

- 12 -

These factors could be readily adjusted by one skilled in the art to obtain a desired result.

Therapeutic Use

As shown schematically in FIG. 4, a patient requiring immunotherapy has a small volume of blood drawn. This blood is then used as described above to produce a pool of autologous or allogeneic non-fetal hemangioblasts, which is administered, using standard methods, to the patient as an immune system booster prior to a treatment damaging the patient's immune system and/or blood forming system (e.g., chemotherapy), and/or as a stimulant to the patient's compromised immune or blood forming system after the treatment. Cells are administered to reconstitute portions of the immune system, e.g., bone marrow, using methods described in the literature, e.g., in U.S. Patent Nos. 5,130,144, 5,635,386, 5,670,147, 5,646,043, 5,635,387 and 5,061,620, the disclosures of which are incorporated herein by reference.

Alternatively, a cell sample can be used to produce a pool of a selected population of cells, by first forming a population of non-fetal hemangioblasts and then placing the cells in a culture under conditions selected to promote the proliferation of a desired population of cells using the non-fetal hemangioblasts as precursors.

Newly formed endothelial cells differentiated from the hemangioblasts can be used in various cell therapies. For example, the cells can be used in wound healing, e.g., donor endothelial cells delivered by injection have been found to localize exclusively to neovascular zones of recipient mice. T. Asahara et al., Isolation of Putative Progenitor Endothelial Cells for Angiogenesis, Science, Vol. 275, p. 964, Feb. 14, 1997. Endothelial

- 13 -

cells can also be used in gene therapy, for example endothelial cells can be delivered to inhibit vascularization of tumors, e.g., using switchable genes encoding angiogenesis-inhibiting proteins as disclosed in 5 U.S. Patent Nos. 5,733,876, 5,712,291 and 5,698,586, the disclosures of which are incorporated herein by reference. An *in vitro* production system would be particularly useful for angiogenic activity assays used in cancer detection, screening of anti-angiogenesis 10 agents for cancer therapeutics, and for graft tissue engineering applications, e.g., grafts for neo-vascularization in the treatment of eschemic cardiovascular diseases, including coronary artery, peripheral artery and cerebral vascular.

15 EXAMPLE 1

Growth Medium: Iscove's Modified Dulbecco's Medium (1MDM) (100 ml), Pen/Strep (50 μ l), BSA (50 mg/ml), Insulin (50 μ g/ml), Transferrin (1 mg/ml), Low Density Lipoprotein (100 μ l), 2-Mercapto-Ethanol (7 μ l of 1/100 20 solution), FLT3 (100 ng/ml), SCF (100 ng/ml), Tpo (100 ng/ml).

1. Obtained an Umbilical Cord Blood extract containing Lin⁻ cells, including CD 34-, Lin⁻ cells.
2. Separated the Mononuclear Fraction (MNF) by 25 Ficoll Density Gradient Centrifugation.
3. Separated Lin⁻ cells from the MNF using negative selection.
4. Inoculated a culture plate containing 10ml of the above growth medium with 5,000 Lin⁻ cells/ml, using 30 the cells from step 3.

- 14 -

5. Placed the culture plate in an incubator at 37°C and 5% CO₂.

6. On day 7, harvested the culture and purified by negative (-) selection, as described below, for Lin⁻ 5 cells.

7. Inoculated fresh growth medium with the Lin⁻ cells obtained in step 6, at a concentration of 5,000 cells/ml, and placed the culture plate in an incubator at 37°C and 5% CO₂.

10 8. Multiple cycles of reselection, using the specified medium for interim growth, were performed on days 7, 14 and 21.

Negative (-) Selective Procedure (Step 6 above):

We incubated the cells in the culture with a 15 cocktail containing selection molecules (antibodies to surface antigens) linked to an anti-dextran molecule. After incubation, a magnetic dextran iron particle colloid was introduced into the cell suspension. A Cell/Antigen/Antibody/Anti-dextran/Dextran/Iron Complex 20 formed. This complex was then passed through a magnetic field, removing cells not of the predetermined target population from the nutrient medium. The target population was collected downstream and returned to the nutrient medium. The detailed procedure we followed is 25 described below.

1. A sample of human cord blood was obtained.

2. A Mononuclear Cell Composition (Buffy Coat) using Ficoll-Paque (Pharmacia Biotech) Gradient Centrifugation was prepared and rinsed twice with PBS 30 (without Ca++ or Mg++).

3. 100 µl of antibody cocktail containing bispecific Tetrameric Antibody Complex: Anti-Dextran/Anti- CD3, CD2, CD56, CD24, CD19, CD66b, CD14,

- 15 -

CD16 and Glycophoran A (StemSep) was added to 1 ml containing 2×10^7 Mononuclear Cells obtained in Step 2 above.

4. This mixture was then incubated for 15 minutes
5 at room temperature.

5. 60 μ l of colloidal magnetic dextran iron particles (StemSep) was added.

6. This mixture was incubated for 15 minutes at room temperature.

10 7. During the incubation steps above, a 0.6" diameter separation column (StemSep), pump (Cole-Parmer), and magnet (StemSep) were assembled and the flow rate calibrated according to the column manufacturer's specifications.

15 8. Once the column was in place within the magnetic field and fully primed with PBS (without FBS) the sample was loaded into the top of the column.

9. The pump was started in the downward direction to allow the sample to run into the matrix of the column.

20 10. PBS plus FBS was added to the top of the column intermittently so as not to allow the sample front to enter the column matrix until 25 mls were collected downstream of the magnet.

11. The 25 ml sample containing the target
25 population was centrifuged at 800 RPM for 15 minutes.

12. The resulting pellet was resuspended in 1 ml of HBS and transferred to a 1.5 ml eppendorf tube.

13. The sample was rinsed twice with repeated centrifugation at 800 RPM and resuspended in 1 ml HBS.

30 14. Following analysis by flow cytometry the target cells were plated in the growth medium at a concentration of 1000 cells/ml.

Data obtained from the cultures described above is shown in Figs. 5-9.

- 16 -

Fig. 5 is a graph showing the growth kinetics of CD34+/CD38-/Lin- and CD34-/CD38-/Lin- target cells over a 30 day culture period. As shown in Fig. 5, the population of CD34-, Lin- cells continued to expanded throughout the 5 30 day period.

Fig. 6 is a group of dot plots showing the population profile of reselected cells prior to subsequent reselection using CD34 vs. Lineage (Lin) markers over a three week period. We observed (a) 10 continual output of lineage positive progeny prior to each selection step; (b) continual amplification of CD34+, Lin- cells, and (c) continual amplification of CD34-, Lin- cells.

Fig. 7 is a dot plot showing the population 15 profile of initial negatively selected target populations used to inoculated cultures. Using CD34 vs. anti-VEGF receptor (VEGF_R) we observed the presence of CD34-/VEGF_R+ cells in the inocula (see the circled portions of Fig. 7).

Fig. 8 is a group of dot plots showing the population profile of reselected cells at week three of culture using CD45 vs. MHC I and MHC II and CD34 vs. Flk-1. Virtually all cells in this culture are CD45+ and a vast gradient exists in MHC expression. Importantly, the 25 fact that the cells are CD45+ demonstrates that CD34+/-, Flk-1+ cells are not contaminating Human Umbilical Cord Vascular Endothelial Cells (HUEC), which are CD45-. The gradient in MHC expression demonstrates the presence of both naive and mature leukocytes.

Fig. 9 is a group of dot plots showing the population profile of reselected cells at week three of 30 selective culture using CXCR4 vs. Flk-1 and Pgp (MDR) vs. Flk-1. The circled cells were CD34+ (backgated analysis) and the cells indicated by rectangles were

- 17 -

CD34-/CXCR4+/Flk+ and CD34-/Pgp+/Flk+ populations. Notably, CXCR4 is a chemokine receptor responsible for trafficking engrafting cells into the bone marrow. Also, Pgp is the receptor for the Multi-Drug Resistance (MDR) gene product- that corresponds to Hoechst Lo, which has been correlated with CD34- cells in the mouse and rhesus that have high engraftment potential.

In combination these observations demonstrate the ex vivo production of clinically relevant numbers of CD34+ and CD34-/CD45+/CD38-/Lin-/Flk+/MDR+/CXCR4+ target cells. This development represents a pivotal contribution to the art, allowing the ex vivo production of rare cells with extraordinary utility in the field of cellular therapeutics.

15 Other embodiments are within the claims.

What is claimed is:

- 18 -

CLAIMS

1. A method for providing a cell population containing non-fetal hemangioblasts, said method comprising
 - 5 a) providing a cell population containing non-fetal hemangioblasts; and
 - b) growing said cell population under conditions that promote the proliferation of said non-fetal hemangioblasts.
 - 10 2. The method of claim 1 wherein, in step (b), said conditions are such that the number of said non-fetal hemangioblasts and their proximity to each other are sufficient to increase the proportion of non-fetal hemangioblasts in the population, compared to other 15 cells, to provide an enriched cell culture.
 3. The method of claim 1 further comprising (c) separating at least a portion of said non-fetal hemangioblasts from other cells in said cell culture.
 4. The method of claim 3 wherein said separating 20 step (c) comprises a negative selection process.
 5. The method of claim 3 wherein said separating step (c) is performed concurrently with, intermittently during, or following, said growing step (b).
 6. The method of claim 3 wherein said separating 25 step is performed more than once during growing step (b).
 7. The method of claim 6 wherein said separating step is performed every 5 to 10 days.

- 19 -

8. The method of claim 1 wherein said growing step includes providing at least one growth factor to said cell population during cell proliferation.

9. The method of claim 8 wherein a plurality of 5 growth factors is provided to said cell population.

10. The method of claim 9 wherein said growth factors comprise SCF, TPO and FLT-3.

11. The method of claim 8 wherein said growth factor is VEGF.

10 12. The method of claim 1 wherein said non-fetal hemangioblasts are human hemangioblasts.

13. The method of claim 1 wherein the non-fetal hemangioblasts provided in the providing step (a) are obtained from human cord blood.

15 14. The method of claim 12 wherein at least 5% of the non-fetal hemangioblasts are said human hemangioblasts.

15. The method of claim 14 wherein at least 15% of the non-fetal hemangioblasts are said human
20 hemangioblasts.

16. The method of claim 15 wherein at least 25% of the non-fetal hemangioblasts are said human hemangioblasts.

17. The method of claim 12 wherein said human
25 hemangioblasts are characterized as: CD2⁺, CD3⁺, CD14⁺, CD16⁺, CD19⁺, CD24⁺, CD56⁺, CD66b⁺, glycophorin A⁺.

- 20 -

18. The method of claim 17 wherein said human hemangioblasts are further characterized as: flk-1⁺, CD45⁺.

19. The method of claim 18 wherein said human hemangioblasts are further characterized as: CXCR4+, MDR+.

20. The method of claim 1 wherein at least some of the non-fetal hemangioblasts are CD 34-, Lin- cells.

21. The method of claim 20 wherein the percentage of cells that are CD 34-, Lin- is higher after the growing step (b) than in the starting cell culture.

22. The method of claim 20 wherein at least 5% of the non-fetal hemangioblasts are said CD 34-, Lin- cells.

23. The method of claim 22 wherein at least 15% of the non-fetal hemangioblasts are said CD 34-, Lin- cells.

24. The method of claim 23 wherein at least 25% of the non-fetal hemangioblasts are said CD 34-, Lin- cells.

20 25. A cell population comprising non-fetal hemangioblasts, the cell population resulting from expansion of a starting cell sample containing fewer non-fetal hemangioblasts than the cell population.

25 26. The cell population of claim 25 wherein said starting cell sample contains a proportion of non-fetal hemangioblasts at least 10% lower than the cell population.

- 21 -

27. The cell population of claim 25 wherein the percentage of cells that are non-fetal hemangioblasts in the enriched cell culture is higher than the percentage of cells that are non-fetal hemangioblasts in the
5 starting cell sample.

28. A composition of cells in which at least 2% of the cells are non-fetal hemangioblasts.

29. The composition of claim 28 wherein at least 5% of the cells are said non-fetal hemangioblasts.

10 30. The composition of claim 29 wherein at least 15% of the cells are said non-fetal hemangioblasts.

31. The composition of claim 30 wherein at least 25% of the cells are said non-fetal hemangioblasts.

32. The composition of claim 28 wherein said non-
15 fetal hemangioblasts comprise human hemangioblasts.

33. The composition of claim 28 wherein said non-fetal hemangioblasts comprise CD 34⁺, Lin⁻ cells.

34. The composition of claim 32 wherein said
human hemangioblasts are characterized as:
20 CD2⁺, CD3⁺, CD14⁺, CD16⁺, CD19⁺, CD24⁺, CD56⁺, CD66b⁺,
glycophorin A⁺.

35. The composition of claim 32 wherein said
human hemangioblasts are further characterized as: flk-
1⁺, CD45⁺.

- 22 -

36. The composition of claim 35 wherein said human hemangioblasts are further characterized as:
CXCR4+, MDR+.

37. A method for providing a cell population
5 containing non-fetal uncommitted human hemangioblasts,
said method comprising
a) providing a first cell population containing
non-fetal uncommitted human hemangioblasts; and
b) growing said first cell population under
10 conditions that promote the proliferation of said non-
fetal human uncommitted hemangioblasts.

Developmental Hierarchy

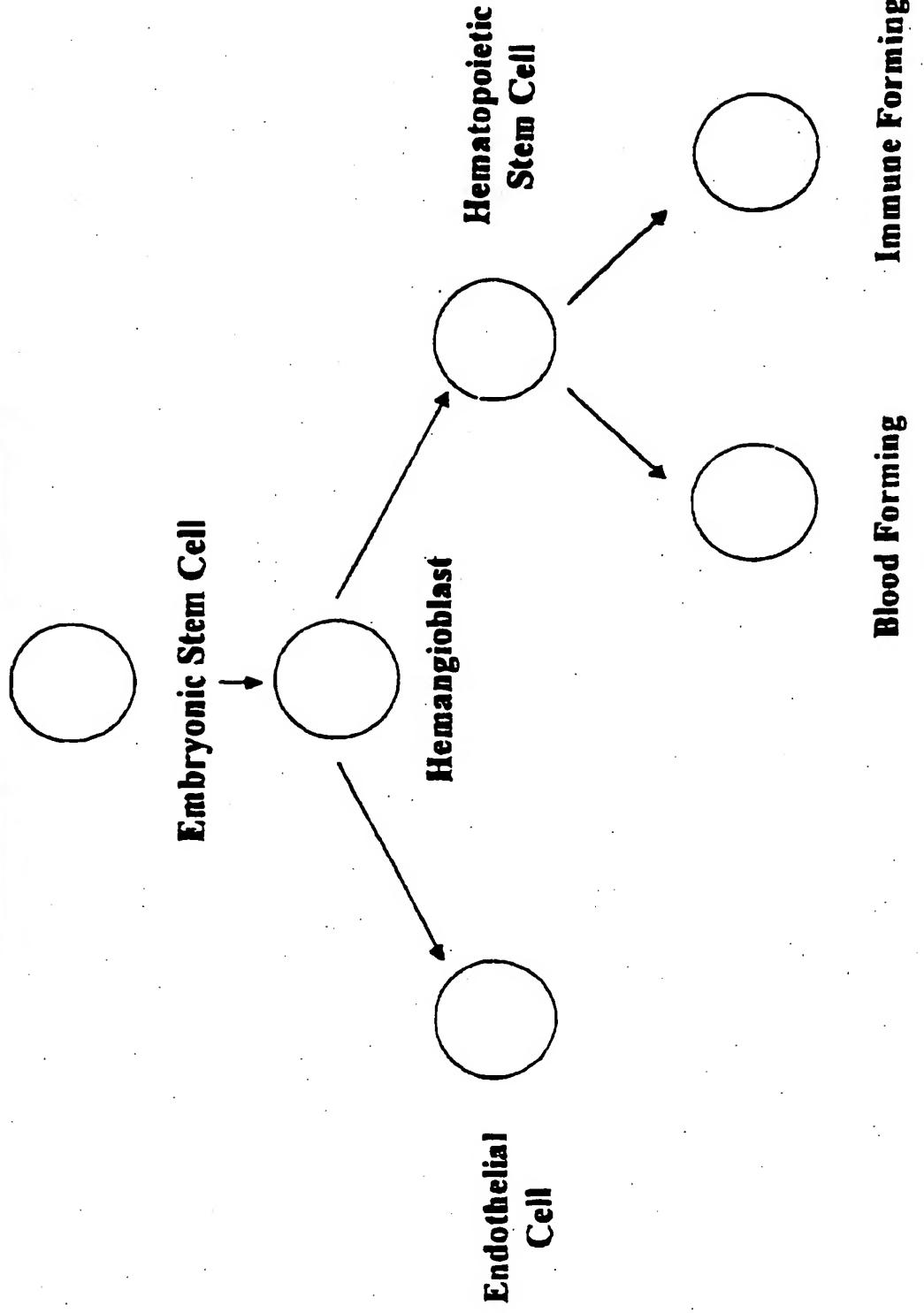
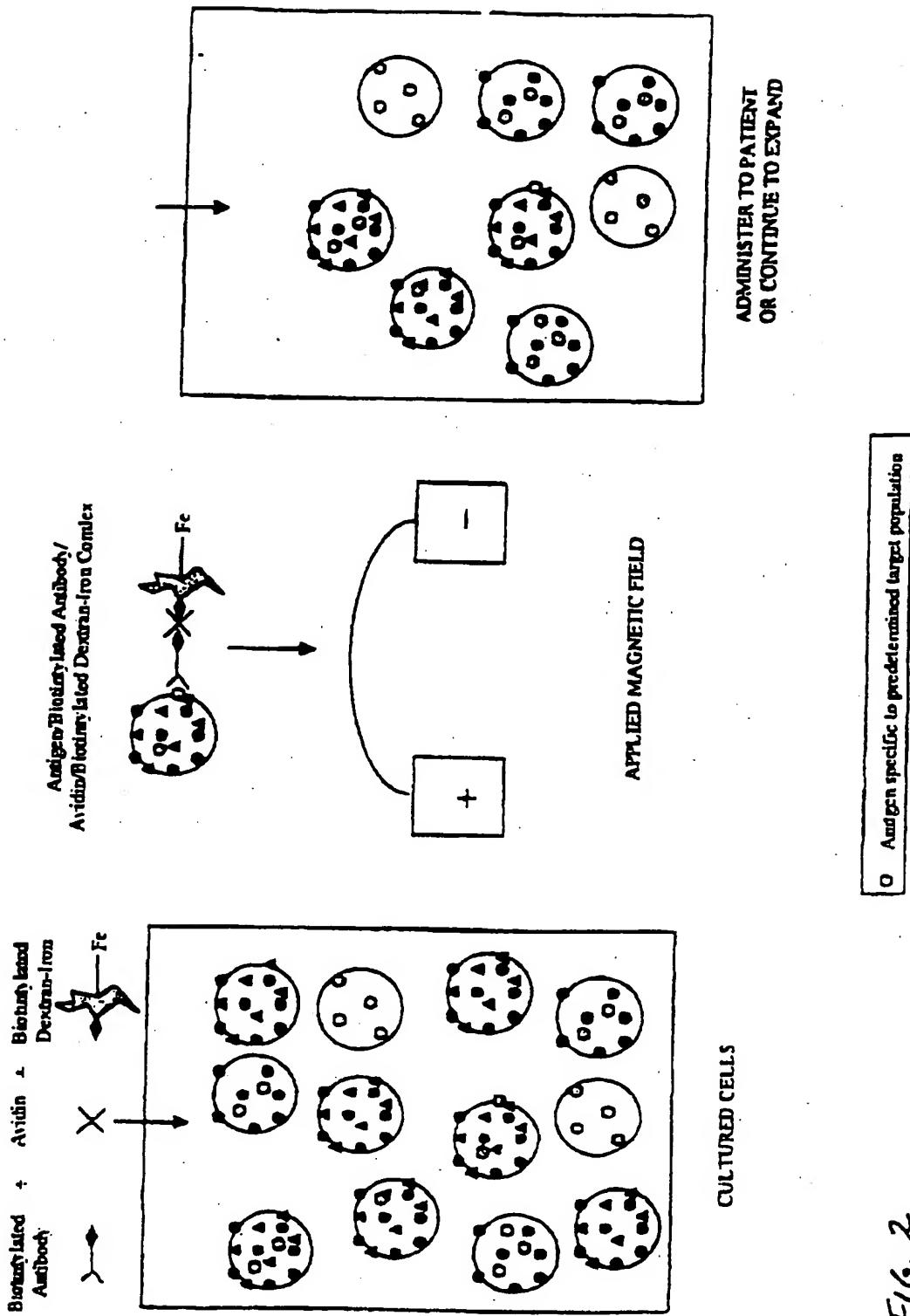


FIG. 1

Positive (+) Selection



F16. 2

Negative (-) Selection

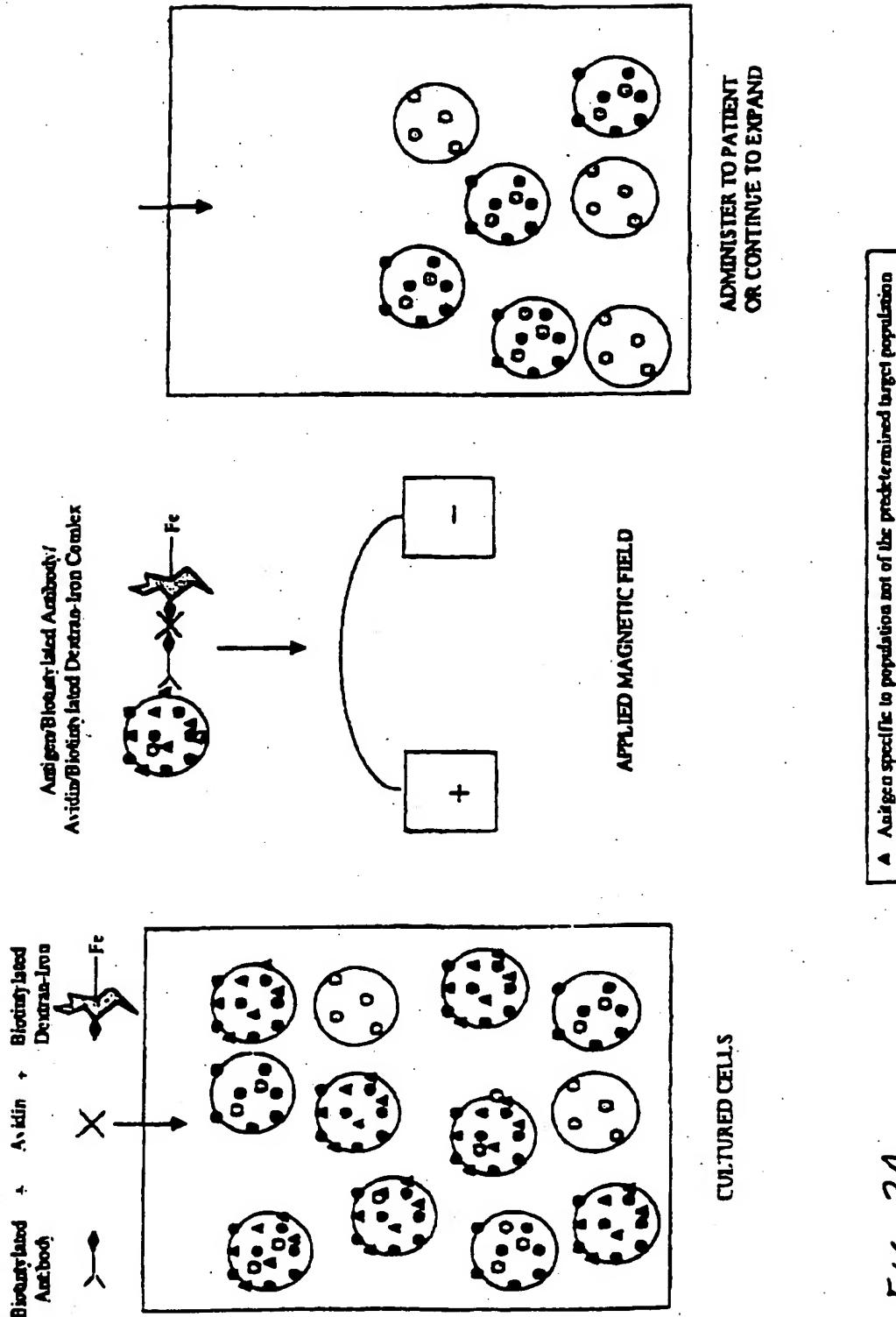


FIG. 2A

4/10

MODES OF OPERATION

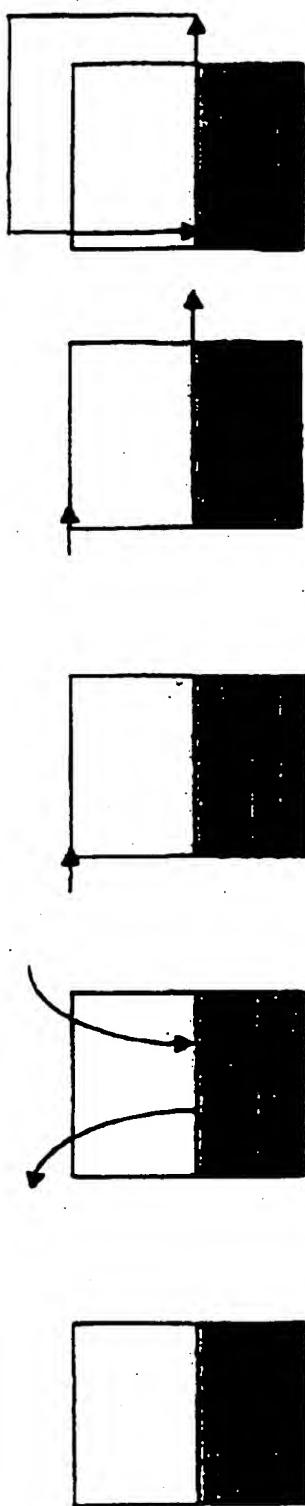


FIG. 3

FIG. 3A

FIG. 3C

FIG. 3D

BATCH SEMI-BATCH FED BATCH CONTINUOUS RECYCLED

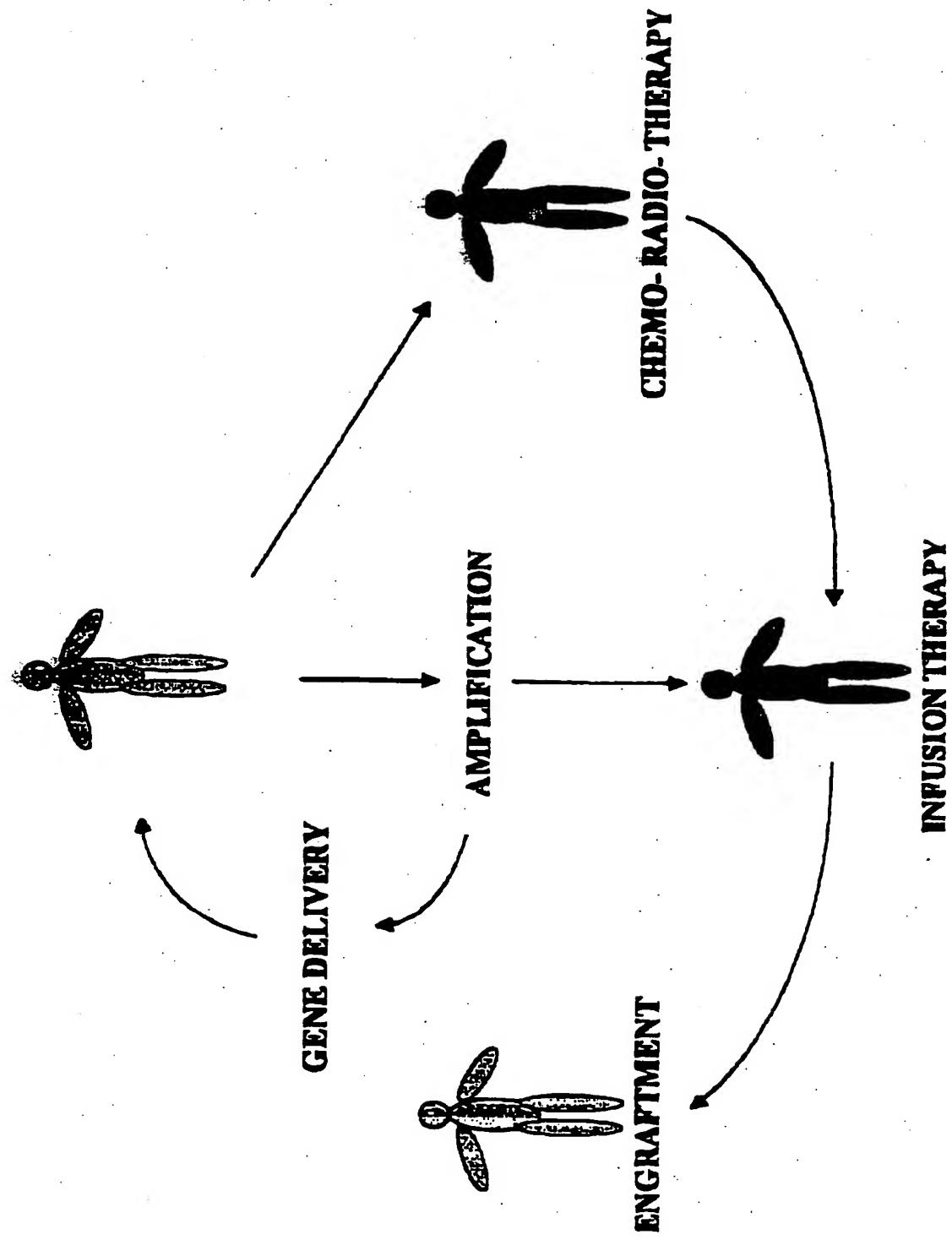


FIG. 4

GROWTH KINETICS FOR CD34+/CD38-/LIN- AND
CD34-/CD38-/LIN- TARGET CELLS

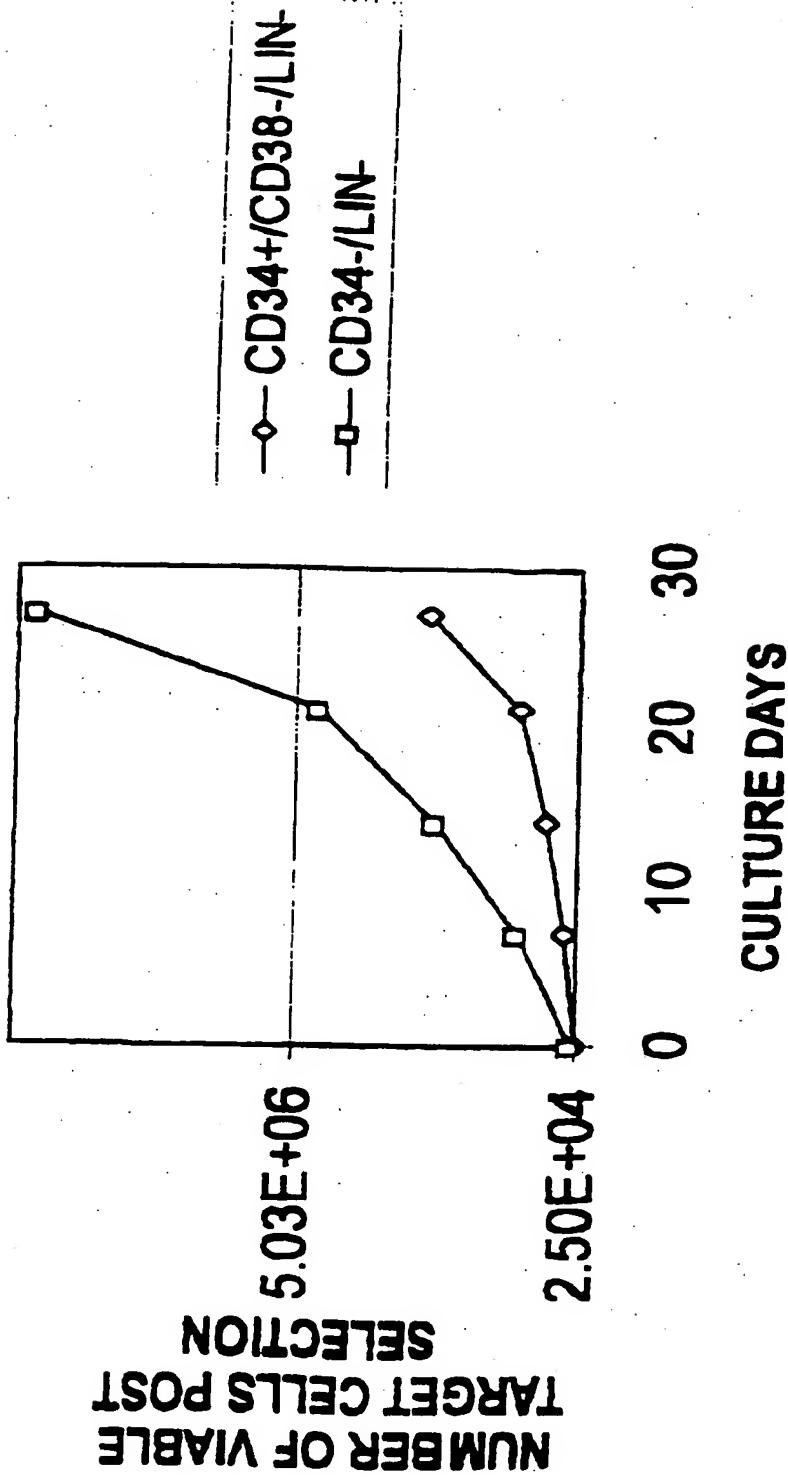
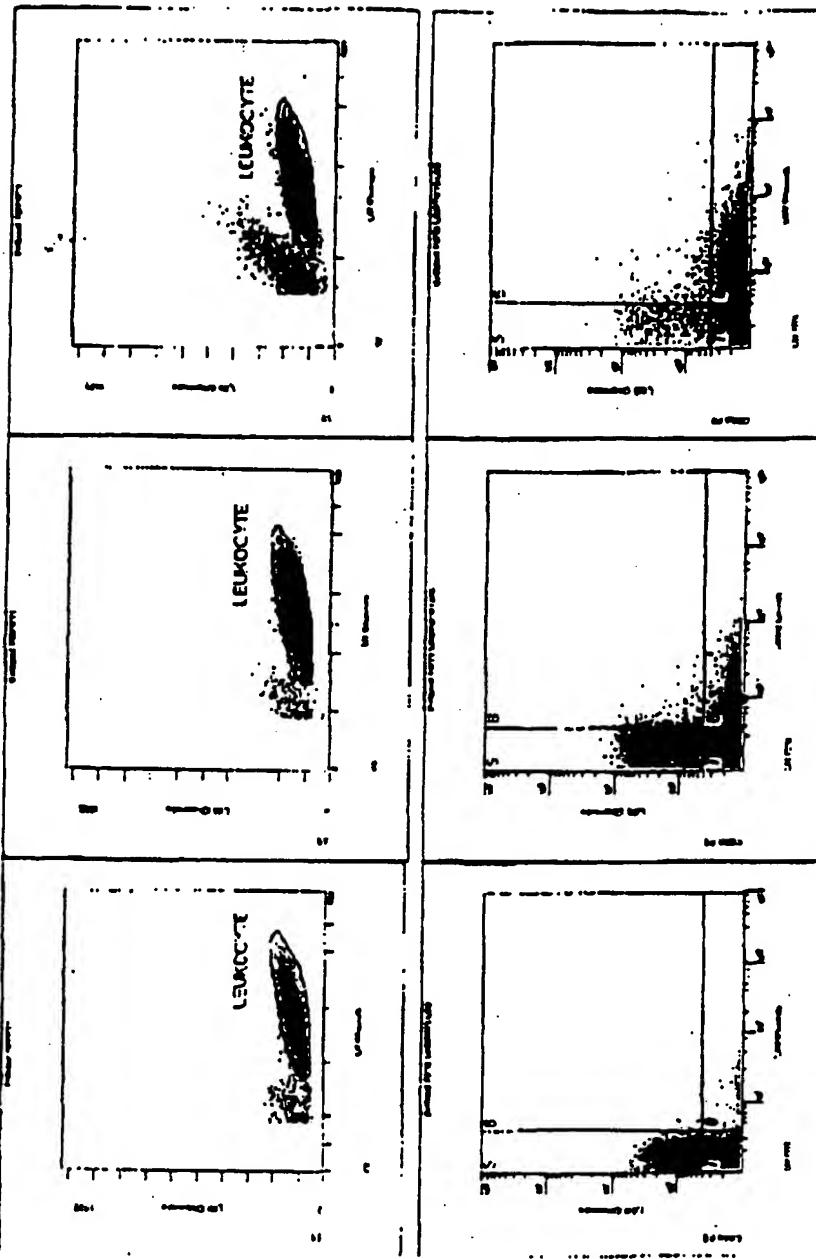


FIG. 5

**Population Profile of Reselected Cells
Prior to Subsequent Reselection
CD34 vs Lineage (Lin)**



Day 0

Week 2

Week 3

FIG. 6

Population Profile of Initial Negatively Selected Target Population
Used to Inoculate Cultures
CD34 vs anti-VEGF Receptor

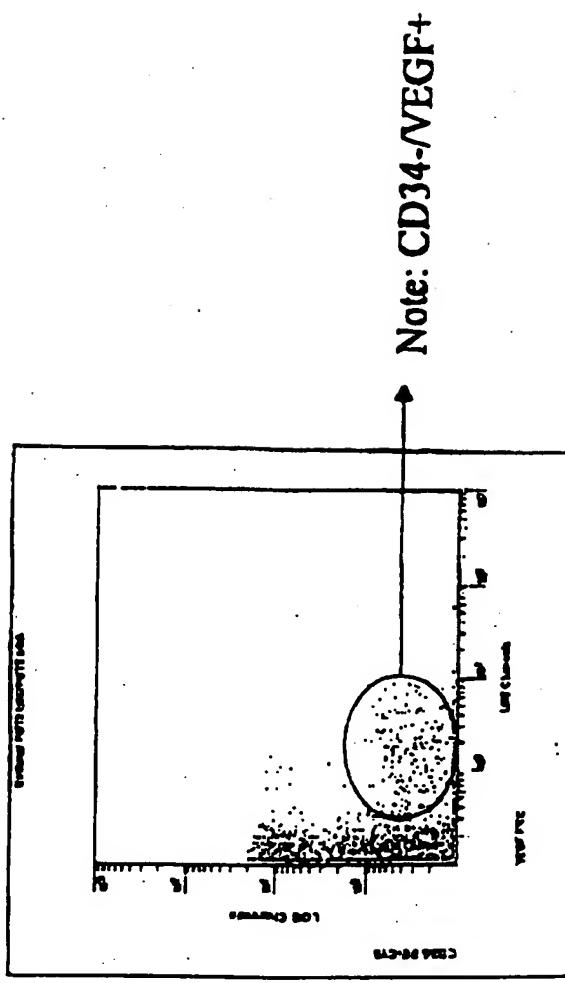
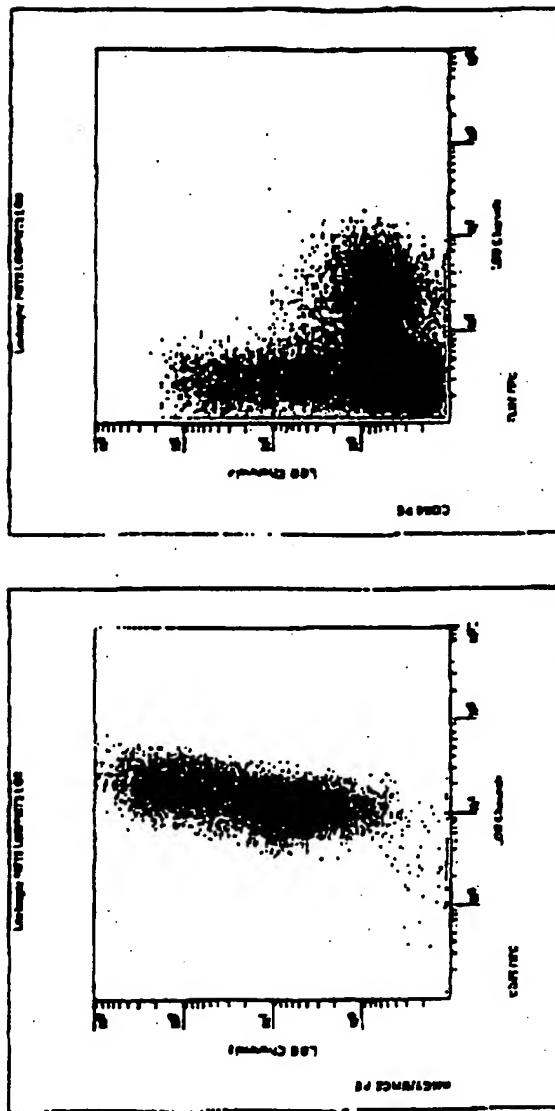


FIG. 7

9/10

Population Profile of Reselected Cells
CD45 vs MHC I & MHC II
CD34 vs Flk-1

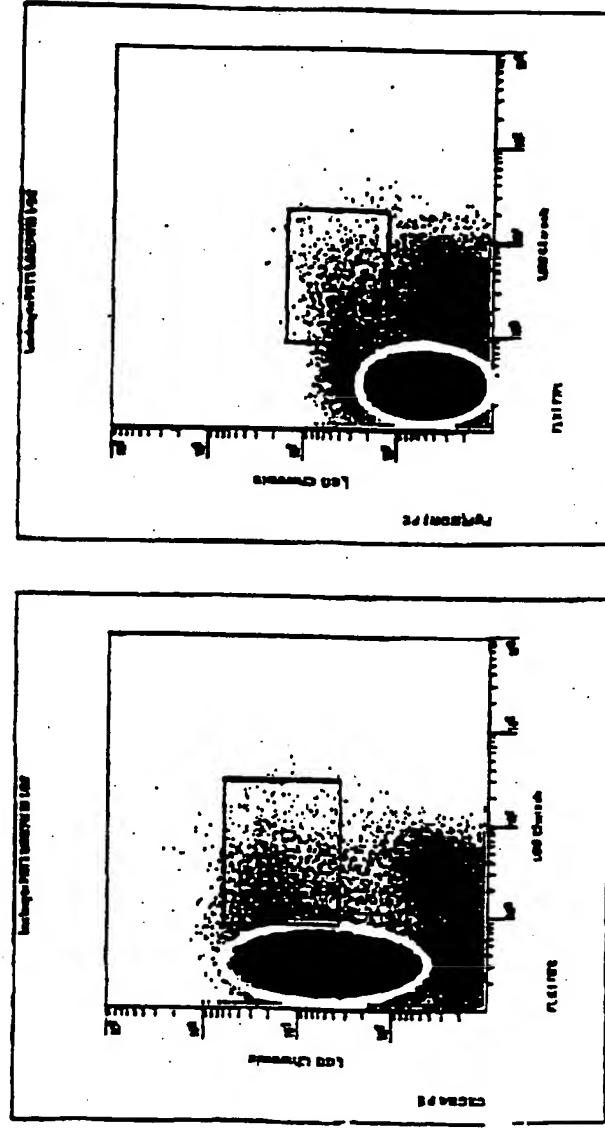


Week 3 of Selective Culture

FIG. 9

FIG. 9

Population Profile of Reselected Cells CXCR4 vs Flk-1 (VEGF) and Pgp (MDR) vs Flk-1 (VEGF)



Week 3 of Selective Culture

Note: Circles indicate location of CD34+ cells. Rectangles indicate the location of CD34-/CXCR4+/Flk+ and CD34-/Pgp+/Flk+ cells, respectively.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/19159

A. CLASSIFICATION OF SUBJECT MATTER IPC 7 C12N5/08 C12N5/00		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 7 C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the International search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	B.Q. SHI ET AL.: "Evidence for circulating bone marrow-derived endothelial cells" BLOOD, vol. 92, no. 2, 1998, pages 362-367, XP002125750 the whole document	1,25,37
Y	A.G. ELEFANTY ET AL.: "Hematopoietic specific genes are not induced during in vitro differentiation of scl-null embryonic stem cells" BLOOD, vol. 90, no. 4, 1997, pages 1435-1447, XP002125751 the whole document	1-37
X	A.G. ELEFANTY ET AL.: "Hematopoietic specific genes are not induced during in vitro differentiation of scl-null embryonic stem cells" BLOOD, vol. 90, no. 4, 1997, pages 1435-1447, XP002125751 the whole document	1,25,37
Y	---	1-37
	-/-	
<input checked="" type="checkbox"/>	Further documents are listed in the continuation of box C.	<input type="checkbox"/> Patent family members are listed in annex.
* Special categories of cited documents :		
<p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>		
Date of the actual completion of the international search	Date of mailing of the international search report	
15 December 1999	30/12/1999	
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fax: (+31-70) 340-3016	Authorized officer Marie, A	

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/19159

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M. TAVASSOLI: "Embryonic and fetal hemopoiesis: an overview" BLOOD CELLS, vol. 17, no. 2, 1991, pages 269-281, XP002125752 the whole document ----	1,25,37
Y		1-37
X	R. AUERBACH ET AL.: "Hematopoietic stemm cells in the mouse embryonic yolk sac" STEM CELLS, vol. 14, 1996, pages 269-280, XP002125753 the whole document ----	1,25,37
Y		1-37
Y	L. ROBB ET AL.: "The hemangioblast-an elusive cell captured in culture" BIOESSAYS, vol. 20, 1998, pages 611-614, XP002125754 the whole document ----	1,25,37
Y		1-37
Y	K. CHOI ET AL.: "A common precursor for haematopoietic and endothelial cells" DEVELOPMENT, vol. 125, 1998, pages 725-732, XP002125755 cited in the application the whole document ----	1,25,37
Y		1-37
Y	A. EICHMANN ET AL.: "Ligand-depenednet development of the endothelial and hemopoietic lineages from embryonic mesodermal cells expressing vascular endothelial growth factor receptor 2" PNAS, vol. 94, 1997, pages 5141-5146, XP002125756 the whole document ----	1,25,37
Y		1-37